EXHIBIT 1

Recombinant 55-kDa Tumor Necrosis Factor (TNF) Receptor

STOICHIOMETRY OF BINDING TO TNFα AND TNFβ AND INHIBITION OF TNF ACTIVITY*

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The extracellular domain of the 55-kDa TNF receptor (rsTNFRβ) has been expressed as a secreted protein in baculovirus-infected insect cells and Chinese hamster ovary (CHO)/dhfr⁻ cells. A chimeric fusion protein (rsTNFRβ-hγ3) constructed by inserting the extracellular part of the receptor in front of the hinge region of the human IgG Cγ3 chain has been expressed in mouse myeloma cells. The recombinant receptor proteins were purified from transfected cell culture supernatants by TNFα- or protein G affinity chromatography and gel filtration. In a solid phase binding assay rsTNFRβ was found to bind TNFα with high affinity comparable with the membrane-bound full-length receptor. The affinity for TNFβ was slightly impaired. However, the bivalent rsTNFRβ-hγ3 fusion protein bound both ligands with a significantly higher affinity than monovalent rsTNFRβ reflecting most likely an increased avidity of the bivalent construct. A molecular mass of about 140 kDa for both rsTNFRβ·TNFα and rsTNFRβ·TNFβ complexes was determined in analytical ultracentrifugation studies strongly suggesting a stoichiometry of three rsTNFRβ molecules bound to one TNFα or TNFβ trimer. Sedimentation velocity and quasielastic light scattering measurements indicated an extended structure for rsTNFRβ and its TNFα and TNFβ complexes. Multiple receptor binding sites on TNFα trimers could also be demonstrated by a TNFα-induced agglutination of Latex beads coated with the rsTNFRβ-hγ3 fusion protein. Both rsTNFRβ and rsTNFRβ-hγ3 were found to inhibit binding of TNFα and TNFβ to native 55- and 75-kDa TNF receptors and to prevent TNFα and TNFβ bioactivity in a cellular cytotoxicity assay. Concentrations of rs-TNFRβ-hγ3 equimolar to TNFα were sufficient to neutralize TNF activity almost completely, whereas a 10–100-fold excess of rsTNFRβ was needed for similar inhibitory effects. In view of their potent TNF antagonizing activity, recombinant soluble TNF receptor fragments might be useful as therapeutic agents in TNF-mediated disorders.

Tumor necrosis factor (TNF)[1] α and β are two cl sely related cytokines with about 30% sequence homology (1–3). Their genes are closely linked to the major histocompatibility complex of mammals (4). TNFα and TNFβ are primarily produced by activated macrophages and lymphocytes, respectively (5, 6). Based on crystallographic (7, 8) and analytical centrifugation studies (9) both cytokines are believ d to form trimers. A wide variety of TNFα and TNFβ activities in vitro has been described including growth enhancement of fibroblasts (10), growth inhibition or lysis of some transformed cells (11), differentiation of human myeloid cell lines (12), and induction of the expression of cell surface molecules (13–15). In vivo TNFα induces hemorrhagic necrosis of certain transplantable tumors (16, 17), is involved in immune and inflammatory reactions (18–20), and mediates lethal effects in endotoxin-induced septic shock (21–23).

We have identified two human TNF receptors of about 75- and 55-kDa apparent molecular masses (in the present paper called TNFRα and TNFRβ, respectively) by chemical cross-linking with radiolabeled TNFα (24) and by binding of m no-clonal antibodies generated against isolates of the receptors (25). Subsequently, both receptors have been purified from HL60 cells and partial amino acid sequences were determined (26). More recently, the cDNAs of TNFRα and TNFRβ were isolated by us (27, 28) and several other groups (29–34). The two receptors show similar cysteine-rich motifs in their extracellular domains and belong to a new cytokine recept r gene family which includes the nerve growth factor receptor, CD40, and OX40 antigens (28, 35).

Soluble fragments of both TNF receptors have been f und to be present in human serum and urine (36–40). In certain disease states receptor shedding appears to be increased (40, 41). Soluble TNF receptors have also been identified in cell culture medium of some transformed cell lines (32, 42) and of stimulated polymorphonuclear leukocytes (43). In functi nal studies the natural TNF receptor fragments have been shown to protect cells from TNFα-induced cytotoxicity (36–39) and, in a recent report, to prevent TNFα-induced hemorrhagic necrosis of a transplanted Meth A sarcoma in BALB/c mice (40). The TNF-antagonizing effects of the soluble receptor fragments in vitro and in vivo imply a specific interaction with TNFα and TNFβ which might be an important regulatory mechanism of TNF action.

[1] The abbreviations used are: TNF, tumor necrosis factor; TNFRα. 75-kDa TNF receptor; TNFRβ, 55-kDa TNF receptor; rsTNFRβ. recombinant soluble TNFRβ; rsTNFRβ-hγ3. recombinant soluble TNFRβ-human IgG Cγ3 fusion protein; PBS. phosphate-buffered saline; SDS-PAGE. sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CHO. Chinese hamster ovary.

In the present work a recon.   ⅃.   soluble form of the 55-kDa TNF receptor (rsTNFRβ) was produced in high yields in different eukaryotic expression systems. The rsTNFRβ was also expressed as a human IgG Cγ3 fusion protein (rsTNFRβ-hγ3) in myeloma cells. The recombinant receptor molecules were found to bind stoichiometrically to TNFα and TNFβ trimers and to neutralize TNF bioactivity in different assay systems.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The *Spodoptera frugiperda* (Sf9) cell line was obtained from American Type Culture Collection (ATCC CRL 1711). The baculovirus *Autographa california* (AcNP virus) was obtained from M. Summers, Texas A & M University, the Chinese hamster ovary (CHO)/dhfr⁻ cell line from P. Familetti, Hoffmann-LaRoche Ltd., Nutley, NJ, and the WEHI164 (clone 2A3) cell line from J. R. Frey (51). The mouse myeloma cell line J558L was kindly provided by A. Traunecker, Basel Institute of Immunology. The expression vector used to construct the rsTNFRβ-hγ3 fusion protein was modified from a CD4-immunoglobulin construct obtained from K. Karjalainen and A. Traunecker (44). Recombinant human TNFα and TNFβ and mouse TNFα produced in *Escherichia coli* were kindly provided by W. Hunziker, H.J. Schoenfeld, and E. Hochuli (Hoffmann-LaRoche Ltd.,Basel). Radioiodination of TNFα and TNFβ was performed with Na¹²⁵I and Iodo-Gen (Pierce Chemical Co.) as described (25). For affinity column chromatography TNFα was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) according to the guidelines of the manufacturer. Protein G-Sepharose 4 Fast Flow was purchased from Pharmacia. Latex beads (polystyrene microspheres, 0.48 μm diameter) were originally obtained from Polysciences, Inc., Warrington, PA and kindly provided by R. Spinnler and M. Caravatti (Hoffmann-LaRoche Ltd., Basel, Diagnostic Division).

Construction of Vectors, Expression, and Purification—The cDNA encoding the extracellular domain of TNFRβ, including the signal peptide, was amplified by the polymerase chain reaction. Unique restriction sites were introduced at both ends of the fragment. In addition, a translational stop codon was introduced behind the last amino acid of the extracellular domain (Thr⁺¹⁸², numbering according to Ref. 27). The engineered fragment was cloned into an expression vector for mammalian cells. The plasmid contained the Rous sarcoma virus long terminal repeat and the 3′ intron plus the polyadenylation site from the rat preproinsulin gene. The expression cassette was finally inserted into the PvuII restriction site of plasmid pSV2-DHFR. Transfected CHO/dhfr⁻ cells were initially selected by the neomycin analogue G418 in α-medium containing 200 nmol/ml methotrexate. Thereafter, the concentration of methotrexate was sequentially increased by 2–5-fold increments up to 150 μmol/ml. For expression in the baculovirus system, homologous recombination was used to introduce the amplified cDNA fragment into the genome of the AcNP virus. Sf9 cells were grown at 27 °C in EX-CELL 400 medium (J. R.Scientific, Woodland, CA) containing 2% fetal bovine serum. Cell culture and viral infection were carried out as described (45). The recombinant viruses were purified by limited dilutions in microtiter plates followed by dot blot hybridization. The rsTNFRβ-hγ3 fusion protein was constructed by exchanging the CD4 sequence in the pCD4-hγ3-4 vector (44) with the TNFRβ extracellular domain sequence using Sst restriction sites. This procedure yielded a chimeric protein in which the TNFRβ sequence was inserted in front of the hinge region of the human IgG Cγ3 chain. J558L mouse myeloma cells transfected with the rsTNFRβ-hγ3 construct by protoplast fusion were cultured in DHI medium (Dulbecco's modified Eagle's medium/Ham's F-12/Iscove's modified Dulbecco's medium, 25/25/50) supplemented with selenite (20 nM), ethanolamine (20 μM), insulin (5 μg/ml), human transferrin (6 μg/ml), Primatone RL (2.5 mg/ml), Pluronic F68 (0.1 mg/ml), and 0–2% fetal calf serum (46). Expression of rsTNFRβ and rsTNFRβ-hγ3 was analyzed by a sandwich-type binding assay using radiolabeled or peroxidase-labeled TNFα and the non-neutralizing monoclonal antibody htr-20 (25).

Cell-free supernatants of cell transfectant cultures containing rsTNFRβ or rsTNFRβ-hγ3 were concentrated 5–10-fold by ultrafiltration (molecular mass cutoff of 10 kDa) and clarified by centrifugation and filtration through a 0.45-μm filter. The clear filtrate was applied to a TNFα affinity column (Sf9 and CHO/dhfr⁻ supernatants) or protein G affinity column (J558L supernatants). After extensive washing with phosphate-buffered saline (PBS) the columns

were eluted with   ⅃.⁻ ₌.ɣcine. 100 mM NaCl, pH 2.6. buffer. The fractions containing the recombinant proteins were concentrated and subjected to gel filtration chromatography on TSK3000SW or Superose 12 (Pharmacia) columns with PBS as solvent. The amount of protein was determined by amino acid analysis or BCA assay (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (47) using the mini-gel system of Bio-Rad.

Binding Assay and Scatchard Analysis—A 96-well microtiter plate coated with the TNFRβ-specific non-neutralizing monoclonal antibody htr-20 (25) was incubated with 10 ng/ml rsTNFRβ or rsTNFRβ-hγ3 in 1% defatted milk powder for 3 h at room temperature. Under these conditions only about 10% of the total binding sites were occupied by the receptor protein as determined from a tiration curve (low density packing). In some experiments the antibody-coated plate was incubated with 3 μg/ml soluble receptor to saturate all receptor binding sites (high or maximum density packing). After washing with PBS the wells were incubated with different concentrations of radiolabeled TNFα or TNFβ (1–250 ng/ml) in the presence or absence of a 200-fold excess unlabeled ligand for 16 h at 4 °C. The radioactivity bound to single wells was directly counted in a γ-counter. Nonspecific binding was subtracted. K_d values were determined from Scatchard plots.

Quasielastic Light Scattering and Ultracentrifugation Analysis—Quasielastic light scattering experiments were performed with the system ALV-300 (ALV Laservertriebsges m.b.H., Langen, Germany). Samples of 300 μl were filtered through 0.2-μm filters in closed cylindrical quartz cells. The protein concentration was 0.5–1 mg/ml. Correlation functions were analyzed with the program CONTIN (48) that yields a distribution of relaxations. Mean values for the diffusion coefficient D were calculated assuming either an extended, *i.e.* rod-like (0 moment of the observed distribution) or roughly spherical structure (3rd moment) of the particles.

A Beckman Model E centrifuge with a AnD rotor and a 12-mm double sector Epon cell was used for analytical centrifugation studies. The rotor was run at 56,000 rpm in the sedimentation velocity experiments and at 24,000 or 11,000 rpm in the sedimentation equilibrium experiments. All runs were performed at room temperature using aliquots of the solutions investigated by quasielastic light scattering. Relative mole masses were calculated from the observed sedimentation velocities by the Svedberg equation using the mean values of the diffusion coefficients as described above. The partial specific volume of rsTNFRβ was assumed to be 0.68 ml/g taking into account 30% glycosylation (w/w) (49). Alternatively, the molecular masses were also obtained from the sedimentation equilibrium runs by analyzing the absorption as a function of the square radius (50).

Competitive Inhibition of Ligand Binding to Native TNFRβ and TNFRα Holoreceptors—1–2 ng of native TNFRβ and TNFRα purified from HL60 cells (26) were spotted to prewetted nitrocellulose membranes. After blocking with a solution of 1% defatted milk powder, the membrane was incubated with human radiolabeled TNFα or TNFβ (1 pmol/ml) in the presence of different concentrations of rsTNFRβ or rsTNFRβ-hγ3 for 2 h at room temperature. The membrane was then thoroughly rinsed with PBS and counted in a γ-counter.

WEHI164 Cytotoxicity Assay—WEHI164 cells (clone 2A3, kindly provided by J. R. Frey (51)) were cultured in a microtiter plate at 10⁴ cells/well in a RPMI-based medium in the presence of human TNFα or TNFβ and different concentrations of rsTNFRβ or rsTNFRβ-hγ3 for 48 hours at 37 °C. Cell viability was determined by a dye uptake method as described earlier (9).

Agglutination of Latex Beads—5 mg of Latex beads washed with PBS, pH 5.0, buffer and H₂O were incubated with 250 μg of rsTNFRβ-hγ3 in 0.5 ml of PBS, pH 5.0, overnight at 4 °C on a rotating wheel. The beads were then treated with a solution of 1% defatted milk powder to block any remaining binding sites and washed with PBS buffer. To induce agglutination the beads were suspended at 0.2–1.0 mg/ml in PBS, pH 7.4, containing 0.1 mg/ml bovine serum albumin and 0.1% NaN₃. Human TNFα was added at different concentrations and after overnight incubation at room temperature agglutination was analyzed in a light microscope at × 400 magnification.

RESULTS

Expression, Purification, and Ligand Binding Affinities of rsTNFRβ—Sf9 insect cells infected with the recombinant baculovirus secreted 5–10 μg/ml of soluble receptor into the

medium after 3–5 days in cu. _.re. Transfected CHO/dhfr⁻
cells produced up to 30 μg/ml of the recombinant protein
after amplification in the presence of increasing methotrexate
concentrations. The TNFRβ-hγ3 fusion protein was ex-
pressed and secreted in m use myeloma cells with a yield of
about 0.5–1 μg/ml.

The recombinant soluble TNF receptors were purified by
TNFα or protein G affinity chromatography and gel filtration.
SDS-PAGE analysis revealed for the baculovirus expressed
protein three to four discrete bands between 21 and 25 kDa.
When virus-infected Sf9 cells were cultured in the presence
of tunicamycin, however, a single protein species of 21 kDa
was obtained (see Fig. 1) which also was the only TNFα-
reacting band in a ligand blot experiment (not shown). N-
terminal sequence analysis of the glycosylated baculovirus-
produced material revealed a single sequence starting with
Leu⁺¹ of the mature TNFRβ (not shown). rsTNFRβ produced
in CHO/dhfr⁻ cells yielded two bands migrating on SDS gels
at around 28 and 32 kDa. Sequence analysis of this material
confirmed the expected N terminus, but a second N-terminal
sequence starting at Asp⁺¹² was also present in a roughly 1:1
ratio. Interestingly, Asp⁺¹² has previously been found to be
the N terminus of the naturally occurring TNFRβ fragment
(36). The TNFRβ-hγ3 fusion protein was expressed as a
disulfide-linked homodimer indicating an antibody-like struc-
ture of this molecule. As shown in Fig. 1.reduced samples of
baculovirus- or CHO/dhfr⁻-derived rsTNFRβ migrated at a
slightly lower rate on SDS gels. This is most likely due to the
high content of cysteines in these proteins. A similar obser-
vation has been made earlier with the native 55-kDa TNFRβ
purified from HL60 cells (26).

The soluble receptor fragments produced in either expres-
sion system showed a high affinity for TNFα and a slightly
lower affinity for TNFβ (see Fig. 2). The difference in the
apparent K_d values of rsTNFRβ for TNFα and TNFβ was
most prominent with the CHO/dhfr⁻-derived material. This
finding is in contrast to the native cell surface-bound 55-kDa
TNFRβ, which has been shown to bind both TNFα and TNFβ
with about the same affinity, i.e. K_d values of 326 and 351 pM,
respectively (24, 52)). Interestingly, fully deglycosylated rs-
TNFRβ as expressed in baculovirus-infected Sf9 cells in the
presence of tunicamycin displayed similar binding character-
istics as the glycosylated form (data not shown), confirming
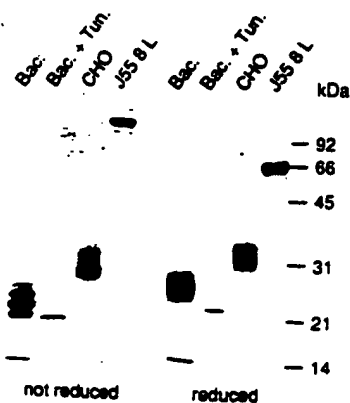that the carbohydrate moieties are not essential for ligand

FIG. 1. **SDS-PAGE analysis of purified rsTNFRβ and rs-
TNFRβ-hγ3.** Purified rsTNFRβ and rsTNFRβ-hγ3 produced in
different expression systems were separated by nonreducing and
reducing SDS-PAGE and stained with Serva blue R. Expression
systems: *Bac.*, baculovirus-infected insect Sf9 cells; *Bac.+Tun.*, bac-
ulovirus-infected insect cells grown in the presence of tunicamycin;
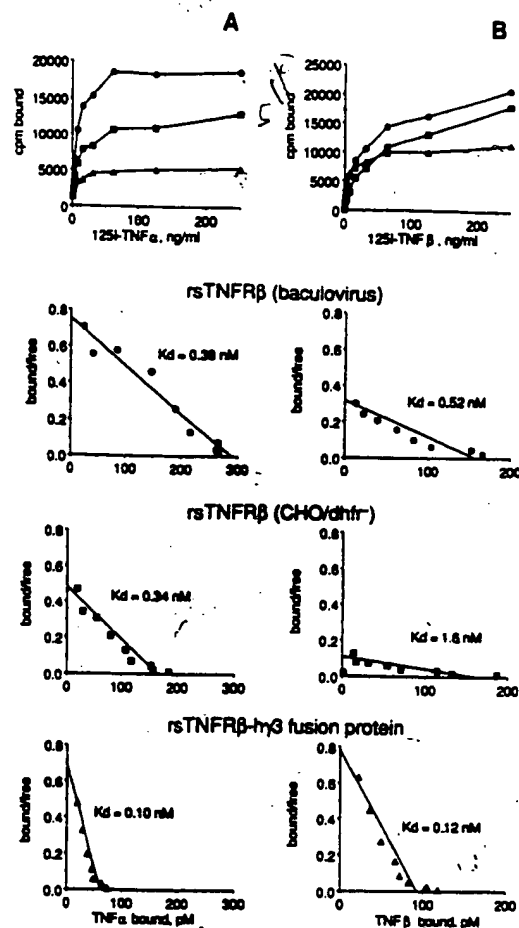*CHO*, CHO/dhfr⁻ cells; *J558L*, mouse myeloma cells (expressing
rsTNFRβ-hγ3).

FIG. 2. **Binding of TNFα and TNFβ to rsTNFRβ and rs-
TNFRβ-hγ3: binding curves and Scatchard analysis.** Binding
of ¹²⁵I-TNFα (A) and ¹²⁵I-TNFβ (B) to baculovirus-produced rs-
TNFRβ (*circles*), CHO/dhfr⁻-produced rsTNFRβ (*squares*), and
rsTNFRβ-hγ3 fusion protein (*triangles*) was measured in a solid
phase assay under low density packing conditions (see "Experimental
Procedures"). The K_d values were determined from Scatchard analysis
of the binding curves as indicated.

binding (24, 26). The apparent affinity of the bivalent rs-
TNFRβ-hγ3 fusion protein for TNFα and TNFβ was found
to be significantly higher than the affinity of baculovirus- or
CHO/dhfr⁻-derived monovalent rsTNFRβ (Fig. 2).It is in-
teresting to note that K_d values determined in the solid phase
assay under high receptor density conditions (see "Experi-
mental Procedures") were generally higher and did not show
a marked difference in the apparent affinities between the
fusion protein and rsTNFRβ (data not shown). It therefore
appears that at maximum dense packing of the solid phase
some interactions of receptor molecules leading to multiple
valency and/or steric constrains cannot be excluded.

*Stoichiometry of rsTNFRβ·TNFα and rsTNFRβ·TNFβ
Complexes*—rsTNFRβ purified from CHO/dhfr⁻ cell culture
medium was incubated with TNFα or TNFβ at different
receptor to ligand molar ratios and fractionated according to
size by gel filtration chromatography. The chromatographic
c nditions chosen allowed to separate receptor-ligand com-
plexes from free receptor and free ligand. As shown in Fig. 3,
at an approximate 1:1 molar ratio neither free receptor nor
free TNFα r TNFβ could be detected in the elution profiles
indicating that under these conditions complete complex for-
mation had occurred. Amino acid c mposition analysis of the
separated complex s evaluated by a recently described com-
puter program (53) confirmed the 1:1 stoichiometry (not

shown). When the amount ι. . α added was gradually increased, a transition of the TNFRβ·TNFα complex toward a slightly lower molecular mass was observed in the elution profile (Fig. 3, *left panel*). In c ntrast, adding increasing amounts of TNFβ did n t affect the elution behavior of the TNFRβ·TNFβ complex (Fig. 3, *right panel*).

To obtain a more accurate molecular mass estimate of rsTNFRβ and its complexes with TNFα and TNFβ, quasielastic light scattering and analytical ultracentrifugation studies were performed. The results are summarized in Table I. For rsTNFRβ a monomeric structure with a molecular mass of 25 kDa was found by sedimentation equilibrium analysis. The theoretical molecular mass for rsTNFRβ in its unglycosylated form is 20,467. Analysis of rsTNFRβ complexed to TNFα or TNFβ under conditions of complete complex for-

FIG. 3. **Analysis of rsTNFRβ and its TNFα and TNFβ complexes by gel filtration chromatography.** 1 nmol of rsTNFRβ purified from CHO/dhfr⁻ cells was mixed with 0.5, 1, 1.5, 2, 3, 4, and 5 nmol of TNFα or TNFβ in 0.1 ml of PBS. (The amount of TNFα and TNFβ was calculated for the 17-kDa monomeric unit.) The mixtures with the various receptor:ligand (*R:L*) molar ratios were fractionated on a Superose 12 column (Pharmacia) in PBS. *Numbers* on *top* indicate the positions of molecular weight marker proteins (Bio-Rad). *Left panel*, TNFα complexes; *right panel*, TNFβ complexes.

mation (see a ., .elded for both complexes a molecular mass of about 140 kDa. If a stoichiometry of three rsTNFRβ molecules bound to one 49-kDa TNFα or 57-kDa TNFβ trimer (9) is assumed, theoretical molecular masses of 124 and 132 kDa, respectively, are calculated which are in approximate agreement with the observed values. Sedimentation velocity analysis combined with quasielastic light scattering data confirmed the molecular masses observed in th equilibrium runs and were, in addition, indicative for a rather extended, *i.e.* rod-like structure of rsTNFRβ and its TNFα and TNFβ complexes.

Inhibition of TNFα and TNFβ Binding by rsTNFRβ and rsTNFRβ-hγ3—rsTNFRβ and rsTNFRβ-hγ3 were tested for their ability to competitively inhibit binding of TNFα and TNFβ to native TNFRα and TNFRβ purified from HL60 cells. In this assay native highly purified receptors were spotted onto nitrocellulose membranes and incubated with ^{125}I-TNFα or ^{125}I-TNFβ in the presence of different concentrations of rsTNFRβ or rsTNFRβ-hγ3. As shown in Fig. 4, *A* and *C*, binding of ^{125}I-TNFα to both TNF receptors was blocked by rsTNFRβ and rsTNFRβ-hγ3 in a concentration-dependent manner. It is interesting to note that a roughly equimolar concentration of the fusion protein was sufficient to prevent TNFα binding almost completely. rsTNFRβ was about 10–100 × less potent in inhibiting the binding. The binding of ^{125}I-TNFβ was also inhibited (Fig.4, *B* and *D*), but higher concentrations of rsTNFRβ and rsTNFRβ-hγ3 were needed to achieve inhibitory effects comparable to TNFα. The 10–15% residual binding seen with iodinated TNFβ at high soluble receptor concentrations is due to nonspecific binding of radioactivity to the nitrocellulose filter.

The inhibition of TNF cytotoxicity by rsTNFRβ and rsTNFRβ-hγ3 was tested in a cellular cytotoxicity assay using the 2A3 subclone of the murine fibrosarcoma cell line WEHI164 (51). As expected from the binding studies, rsTNFRβ-hγ3 very efficiently inhibited TNF activity; at a concentration of 0.1 pmol/ml, *i.e.* equimolar to the TNFα concentration used in the assay, rsTNFRβ-hγ3 prevented TNFα-induced cytolysis very efficiently (Fig. 5A). rsTNFRβ also had inhibitory activity but a concentration about 100-fold in excess of TNFα was needed for complete inhibition. TNFβ-induced cytotoxicity was also inhibited by the fusion protein, albeit not at equimolar concentrations. The protective effects of rsTNFRβ in these cytotoxicity assays were only evident at rather high concentrations (Fig. 5B).

TNFα-induced Agglutination of rsTNFRβ-hγ3-coated Latex Beads—In view of the trimeric structure of TNFα and TNFβ, each capable of binding three recombinant soluble receptor molecules, it is very likely that these cytokines aggregate TNF receptors on the cell surface into microclusters which may be a necessary step in signal transduction. To mimick cell sur-
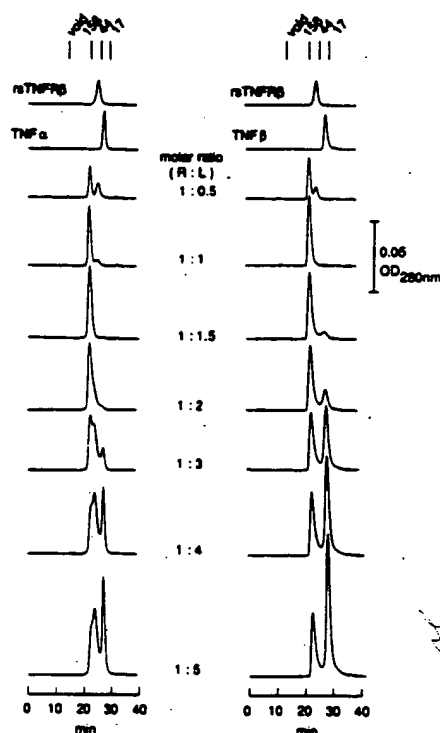
TABLE I

Molecular mass determination of rsTNFRβ and its complexes with TNFα and TNFβ

Molecular masses were determined in the analytical ultracentrifuge as described under "Experimental Procedures." In the sedimentation velocity analyses the molecular masses were calculated according to the Svedberg equation using diffusion coefficients D determined in quasielastic light scattering measurements.

	Molecular mass (kDa)		
	Sedimentation equilibrium	Sedimentation velocity*	Putative stoichiometry
rsTNFRβ	25	20; 32	Monomer
rsTNFRβ·TNFα complex	140	115; 156	[TNFRβ]₃ [TNFα]
rsTNFRβ·TNFβ complex	140	102; 139	[TNFRβ]₃ [TNFβ]

* The 3rd and 0 moments of D were used in the calculation yielding molecular masses for a roughly spherical (listed first) and an extended (listed second) structure, respectively.
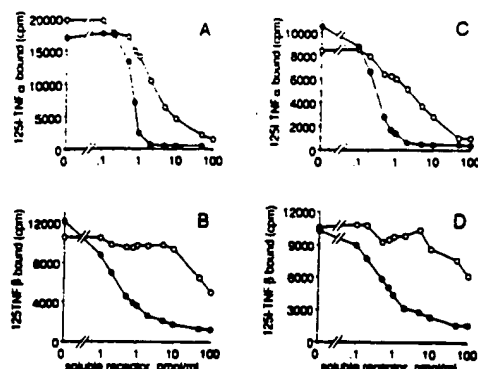
FIG. 4. Binding of ^{125}I-TNFα and ^{125}I-TNFβ to native TNFRβ and TNFRα: inhibition by rsTNFRβ and rsTNFRβ-hγ3. Binding inhibition to native full-length TNFRβ and TNFRα purified from HL60 cells was measured in a dot blot assay as described under "Experimental Procedures." The concentration of radiolabeled ligand in the assay was 1 pmol/ml. *Open circles*, ligand binding in the presence of increasing concentrations of rsTNFRβ; *closed circles*, ligand binding in the presence of increasing concentrations of rsTNFRβ-hγ3. (The concentration of the rsTNFRβ-hγ3 homodimer was calculated for the 66-kDa monomer unit). *A*, ^{125}I-TNFα binding to TNFRβ; *B*, ^{125}I-TNFβ binding to TNFRβ; *C*, ^{125}I-TNFα binding to TNFRα; *D*, ^{125}I-TNFβ binding to TNFRα.
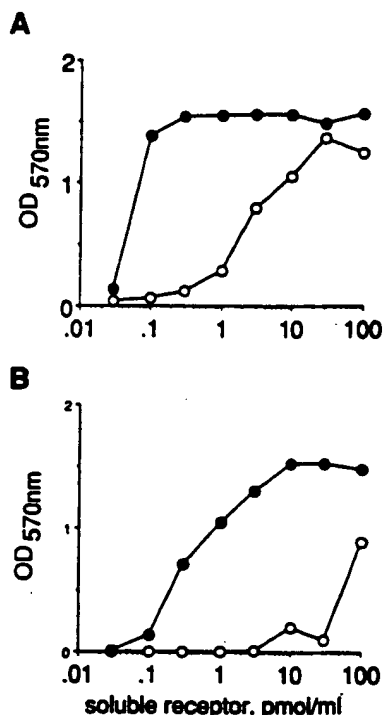

FIG. 5. Inhibition of TNFα- and TNFβ-induced cytotoxicity in WEHI164 cells. WEHI164 cells were cultured in the presence of 0.1 pmol/ml TNFα (*A*) or TNFβ (*B*) and different concentrations of rsTNFRβ (*open circles*) and rsTNFRβ-hγ3 (*closed circles*). Cell viability was analyzed after 48 h at 37 °C.

face-bound TNF receptors, Latex beads were coated with rsTNFRβ-hγ3 fusion protein and subsequently exposed to different concentrations of TNFα. TNFα induced an oligomerization of rsTNFRβ-hγ3 as visualized by agglutination f the Latex beads (Fig. 6). A similar effect was seen with TNFβ, but agglutination was much less pronounced (results not shown).

DISCUSSION

In this study TNF binding and inhibiting properties of the extracellular region f the human TNFRβ were analyzed. The

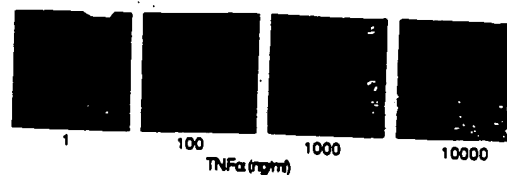FIG. 6. Agglutination of rsTNFRβ-hγ3-coated Latex beads. Latex beads (0.48 μm diameter) were coated with rsTNFRβ-hγ3 and incubated at 1 mg/ml with different concentrations TNFα as indicated. Agglutination of the beads was visualized in a light microscope at × 400 magnification.

recombinant soluble receptors (rsTNFRβ and rsTNFRβ-hγ3 fusion protein) expressed in different eukaryotic expression systems displayed high affinity binding to human TNFα similar to that of native cell surface-bound 55-kDa TNFRβ. In contrast, the binding affinity of rsTNFRβ for TNFβ was significantly decreased when compared with the native cell surface receptor. A similar observation, *i.e.* impaired neutralization of TNFβ *versus* TNFα, has also been made with a so-called TNF binding protein, which is a naturally occurring soluble receptor derived from TNFRβ (33, 36, 39). It therefore appears that with respect to ligand binding properties, rsTNFRβ closely ressembles the natural TNF inhibitor. The apparent lower affinity of rsTNFRβ (and also of the detergent-solubilized holoreceptor (9)) for TNFβ might reflect a microenvironment of the ligand binding site which is slightly different from that of the cell surface-bound full-length TNF receptor. It is noteworthy that with respect to m n valent rsTNFRβ the rsTNFRβ-hγ3 fusion protein binds bothTNFα and TNFβ with a severalfold higher affinity when measured under appropriate assay conditions. This increase in affinity most probably reflects a higher avidity of the rsTNFRβ-hγ3 construct due to its bivalency. Comparison of rsTNFRβ and the fusion protein to compete with native full-length TNF receptors for TNF binding and to protect WEHI 164 c lls from TNF-induced cytotoxicity indeed confirmed the expected higher activity of the fusion protein.

The results from the ultracentrifugation analyses indicate that rsTNFRβ is monomeric in solution. The complexes of rsTNFRβ with TNFα or TNFβ both had a molecular mass of about 140 kDa which favors a stoichiometry of three rsTNFRβ monomers bound to one TNFα or TNFβ trimer. It has been proposed that the receptor binding site on the TNFα trimer is located at the boundary of two monomeric units near the base of the bell-shaped structure thus implying three p tential receptor binding sites (7, 54). Such a model is fully compatible with the size of receptor-ligand complexes as det rmined in the present study. It is interesting to note that an intermediate lower molecular weight form of the rsTNFRβ·TNFα complex can be partially resolved by gel filtration when a slight excess of TNFα over rsTNFRβ is present. Most likely, this intermediate form represents TNFα trimers complexed to only one or two rsTNFRβ molecules. Such intermediate forms are not seen with rsTNFRβ·TNFβ complexes. Whether these distinct binding characteristics of TNFα and TNFβ are also true for cell surface-bound receptors remains to be elucidated.

The results of sedimentation velocity and quasielastic light scattering measurements indicate that rsTNFRβ and its TNFα and TNFβ complexes have a rather extended, *i.e.* rodlike structure. This conclusion is supported by the relative large apparent molecular masses of 62, 170. and 150 kDa for rsTNFRβ, rsTNFRβ·TNFα and rsTNFRβ·TNFβ complexes. r spectively, determined by gel filtration chromatography. A similar relatively large apparent molecular mass (50 kDa) has been found for the natural soluble TNFRβ on sizing columns (42).

Soluble fragments of bo   d and TNFRα are found *in vivo.* They are present at relatively high concentrations in normal human serum and urine but can be drastically increased in certain disease states.[2] The cellular source and the mechanism of receptor shedding remain unclear. It has been speculated that soluble TNF receptor fragments might participate in the control of TNFα and/or TNFβ toxicity by neutralization and rapid clearance of systemic TNFα and TNFβ (33, 36, 37, 39, 42). However, the fact that at least a 10-fold excess of the soluble receptor with respect to TNFα (and more than a 100-fold excess with respect to TNFβ) is needed to obtain a significant neutralization demonstrates that the neutralizing capacity of serum is restricted. The rsTNFRβ-hγ3 construct as described in this study, therefore, is a promising TNF antagonizing agent for neutralization of systemic TNF toxicity in certain disease states.

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